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Bacillus cereus in powdered foods

Characterization of *Bacillus cereus* group isolates from powdered food products

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ABSTRACT

Mashed potato powder as well as powdered infant formula (PIF) are frequently contaminated with *Bacillus cereus sensu lato* (*B. cereus s.l.*), mainly with its spores. These products have also been implicated in foodborne illnesses. Here, we characterized *B. cereus s.l.* isolates originating from powdered products based on sporulation assays, toxin gene profiling, and *panC* typing combined with a SplitsTree analysis. Furthermore, cytotoxicity assays with *B. cytotoxicus* isolates were performed. 78% of PIF tested positive for *B. cereus s.l.*, whereas 92% of all mashed potato powders were positive. In total, 43 isolates were further characterized. The *nhe* and *cytK2* genes were most frequently detected. Moreover, a cereulide-producer was detected from PIF. Most isolates were assigned to *panC* group III, but members of group II, IV, V, and VII could also be found. Nine *B. cytotoxicus* were isolated out of nine mashed potato powders. All *panC* group VII isolates were positive for *cytK1*. Cytotoxicity assays of these nine isolates revealed one highly cytotoxic strain, while all other isolates exhibited no detectable cytotoxicity, underpinning that cytotoxicity of a certain *B. cereus* group strain cannot be deduced from the sole presence or absence of toxin genes.

Keywords: *Bacillus cytotoxicus*; *Bacillus cereus* group; Vero cell assay; mashed potato; powdered infant formula

1. Introduction

Bacillus cereus sensu lato (*B. cereus s.l.*), a group of Gram-positive spore-forming bacteria, is ubiquitous in nature and can therefore widely be found as part of the microflora of agricultural products (Stenfors Arnesen et al., 2008). The group comprises several genetically closely related species, with *B. cereus sensu stricto* as well as *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* as the most prominent members.

B. cereus is known as an important foodborne pathogen that can cause two distinct forms of illness (Stenfors Arnesen et al., 2008). Firstly, the diarrheal syndrome that is linked to three enterotoxins - Hbl, Nhe and CytK - and secondly, the emetic syndrome caused by cereulide toxin preformed in food. *B. thuringiensis* forms characteristic parasporal crystals with insecticidal activity, enabling the use of *B. thuringiensis*-based insecticides in agriculture (Chattopadhyay et al., 2004). *B. thuringiensis* is known as a common contaminant of milk (Bartoszewicz et al., 2008). However, its relevance as a causative agent of foodborne disease has been controversially discussed (EFSA, 2016, Jackson et al., 1995, McIntyre et al., 2008). The thermotolerant species *Bacillus cytotoxicus*, which has been described 2013, characteristically harbors the *cytKI* variant of the cytotoxin K gene (Guinebretière et al., 2013). The description of this novel *B. cereus* group member was based on five strains, four of which were linked to food poisoning, including an outbreak caused by strain NVH 391-98^T that led to three fatalities of diarrheal disease in France in 1998 (Guinebretière et al., 2013, Lund et al., 2000).

The *B. cereus* group species do not show a clear phylogenetic separation and generally form three major clades, in which species are intermingled. SpoAB typing allows the assignment of a strain to a certain clade (Ehling-Schulz et al., 2005; Fricker et al., 2011). For gaining a deeper insight into the population structure of the *B. cereus* group, an AFLP system has been established by Guinebretiere et al. (2008), which allows for assignment of *B. cereus*

group strains to 7 phylogenetic subtypes. *panC* has been found to be a suitable housekeeping gene to assign new strains to these subtypes (Guinebretière et al., 2010). The ability of strains to cause food poisoning was suggested to vary depending on phylogenetic affiliation with *panC* groups I to VII rather than species affiliation (Guinebretière et al., 2010). To date, strains causing emetic illness have exclusively been associated with *panC* group III (Guinebretière et al., 2010).

According to EFSA, *B. cereus* holds fourth place as a cause of foodborne outbreaks in the European Union (EFSA, 2015). It has been stated by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) that *B. cereus s.l.* is an organism of concern in PIF with regard to the strength of evidence of a causal association between the presence of the microorganism in PIF and illness in infants (FAO/WHO, 2006). *B. cereus s.l.* is a frequent contaminant of dried milk products (Becker et al., 1994; Di Pinto et al., 2013; Reyes et al., 2007). Powdered infant formula (PIF) could also represent a source for isolates of the *B. cereus* group, which could have severe consequences as neonates are highly susceptible for infections. *B. cytotoxicus* has been detected in infant foods in China, showing that the possibility of food poisoning outbreaks due to *B. cytotoxicus* is a risk in this particularly vulnerable consumer group (Zhang et al., 2017).

Although the production of powdered products involves heating and drying processes, which pose harsh living conditions for most bacteria, isolates of the *B. cereus* group and in particular *B. cytotoxicus* have mainly been isolated from dehydrated potato products (Contzen et al., 2014) (Kim and Goepfert, 1971; King et al., 2007; Turner et al., 2006). *B. cereus* group isolates are capable of producing spores, which are able to survive stress conditions encountered in the production of powdered products. Foodborne illnesses caused by isolates of the *B. cereus* group in association with potato products have been reported (Doan and Davidson, 2000; Lindqvist et al., 2000). Especially the newly described species *B. cytotoxicus*

that was discovered during an outbreak in France with three fatalities (Lund *et al.*, 2000) has gained attention in recent times. First studies attributed its high cytotoxicity to the possession of *cytKI* (Fagerlund *et al.*, 2007; Lund *et al.*, 2000) and provided phylogenetic data (Guinebretière *et al.*, 2013; Sorokin *et al.*, 2006). Though the number of characterized *B. cytotoxicus* strains is low to date, many of them originated from mashed potatoes and have been linked to food poisoning cases (Guinebretière *et al.*, 2013). A recent study by Contzen *et al.* has shown that *B. cytotoxicus* can frequently be detected in different dehydrated potato products and occurs far more wide-spread than previously suggested (Contzen *et al.*, 2014). Although *B. cytotoxicus* is generally assumed to be highly cytotoxic (Fagerlund *et al.*, 2004; Guinebretière *et al.*, 2010; Hardy *et al.*, 2001), Fagerlund *et al.* suggested that presence of the *cytKI* gene does not correlate with cytotoxic activity (Fagerlund *et al.*, 2007). As cytotoxicity data has so far only been published for three *B. cytotoxicus* isolates (Fagerlund *et al.*, 2007), further cytotoxicity testing is crucial to assess the food poisoning risk related to this new *B. cereus* group species.

Therefore, the objective of the present study was to isolate and characterize *B. cereus* species out of powdered food products including PIF, mashed potato powder, and fruit powder. In addition, we aimed to determine the cytotoxic potential of all isolated *B. cytotoxicus* strains.

2. Materials and methods

2.1 Sampling material and enrichment procedure

A total of 13 powdered mashed potato products and nine PIF from different brands were bought in supermarkets in Switzerland. Furthermore, 11 *B. cereus* group isolates originating from self-control of a powdered infant formula producer were included in the study. In addition, four strains of *B. cereus s.l.* were included in this study that had been isolated out of fruit powders. Two different approaches of enrichment were used for the purchased products. First, 10 g of powder was mixed with 90 ml buffered peptone water (Oxoid, Basel, CH) in a

stomacher bag using the Stomacher® 400 Circulator (Seward, Worthing, UK) for 30 s. The samples were subsequently incubated at 37°C overnight. After overnight incubation, one loop of the overnight culture was streaked onto Mossel (Mossel *et al.*, 1967) and sheep blood agar plates (BD Difco™ Columbia Blood Agar Base) that were incubated at 37°C overnight. Second, an approach was used that has already been described by Contzen *et al.* in order to detect *B. cytotoxicus* (Contzen *et al.*, 2014). This included enrichment of the powder in 90 ml CGY medium (Beecher and Wong, 1994) followed by incubation at 50°C overnight. The next day, a loopful of the enriched culture was streaked onto Mossel and blood agar plates that were subsequently incubated at two different temperatures, 37°C (Mossel) and 50°C (blood agar), respectively. In the present study, the minor modification was made that the culture and the blood plates were incubated at 46°C instead of 50°C. Mossel plates were checked for colonies showing an egg-yolk lecithinase-positive and mannitol-negative phenotype characteristic for isolates of the *B. cereus* group.

2.2 DNA extraction and toxin gene profiling

DNA was extracted from all isolates using the GenElute Bacterial Genomic DNA Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Toxin gene profiles were determined using a PCR approach as previously described by Ehling-Schulz *et al.* (Ehling-Schulz *et al.*, 2006) with minor modifications: The GoTaq PCR system (Promega AG, Dübendorf, Switzerland) was used at (i) 2 min at 95°C, (ii) 30x [45 s at 95°C, 45 s at 51°C, 2 min at 72°C]; (iii) 5 min at 72°C. The respective forward primer used for detection of the *nhe* complex is located in *nheA* while the reverse primer is located in *nheB*, thus enabling detection of the first and second gene of the *nhe* operon. The respective primers for *hbl* are located in *hblD* and *hblA*, thus allowing for detection of the second and third gene of the *hbl* operon. Moreover, a duplex PCR was carried out to distinguish between *cytK1* and *cytK2* as previously described (Guinebretière *et al.*, 2006).

2.3 Genotyping using *panC*

A PCR-based genotyping approach targeting *panC* was performed (Guinebretière et al., 2008). In cases in which previously published *panC* primers did not result in an amplicon, additional primers designed in this study were used (see Table 1). The following cycling conditions were used: (i) 2 min at 95°C, (ii) 30x [45 s at 95°C, 45 s at 60°C, 50 s at 72°C]; (iii) 5min at 72°C. The PCR products were purified with the GenElute™ PCR Clean-Up Kit according to the manufacturer's instructions. Subsequently, the sample's concentration and purity were measured using a NanoDrop™ Fluorospectrometer (Witec AG, Luzern, CH). Sequencing was outsourced (Microsynth™, Balgach, CH). Sequences of *panC* were assigned to seven (I-VII) phylogenetic groups as previously described (<https://tools.symprevious.org/Bcereus/english.php>) (Guinebretière et al., 2008, 2010). Cluster analysis of *panC* sequences was performed with the SplitsTree™ software (<http://www.splitsree.org>). Several reference strains were included in the SplitsTree analysis (*panC* type I: DSM 12442; *panC* type II: WSBC10311; *panC* type III: Ames; *panC* type IV: ATCC 14579; *panC* type V: BCT-7112; *panC* type VI: WSBC 10204; *panC* type VII: NVH391-98).

2.4 Detection of *B. thuringiensis* parasporal crystal

A sporulation assay was performed to identify *B. thuringiensis* isolates. To this end, all isolates were streaked onto T3 plates (Travers et al., 1987), which were incubated for three days at 30°C to promote sporulation. A tiny amount of colony material was mixed with double distilled water on a microscope slide until a homogenous suspension resulted. All strains were checked for the presence of parasporal crystals with diamond, bipyramidal, or spherical shape using a phase contrast microscope (1000 x, oil immersion) (EFSA, 2016).

2.5 Vero cell cytotoxicity assay

A Vero cell assay was used to determine cytotoxicity of all isolated *B. cytotoxicus*. Assays were performed using WST-1 bioassay as described elsewhere (Moravek et al., 2006). Reference strains for low (RIVM Bc90) and high-level toxin production (NVH 0075-95) were included in every run. In order to obtain cell-free culture supernatants, strains were grown in 30 ml CGY broth in an Erlenmeyer flask and were adjusted to an OD₆₀₀ of 0.05 using an overnight culture of the isolate. The day cultures were incubated at 30°C (120 rpm shaking) until an OD of 7 was reached. After centrifugation at 11000 rpm for 10 min and filtration through 0.2 µm sterile filters, aliquots of 1 ml supernatants were supplemented with 10 uL 0.1 M Na₂ EDTA and stored at -80°C.

3. Results

3.1 Identification of *B. cereus* group species and toxin profiling

We detected *B. cereus s.l.* in 78% of purchased PIF and 92% of mashed potato powders. In total, 28 strains were isolated out of the purchased PIF and mashed potato samples. Six products harbored *B. cereus s.l.* of two or more different colony morphologies on blood agar. Including the 11 strains provided by a PIF producer and the four strains that originated from fruit powder, a total of 43 strains have been characterized.

Parasporal crystals were detected in one of the 43 isolates (P21), which exhibited small, round-shaped crystals and originated from powdered infant formula. Nine isolates were classified as *B. cytotoxicus* based on presence of *cytK1* and their affiliation to *panC* group VII. These isolates originated from mashed potato powder from nine different brands. An overview of all other toxin genes detected by PCR is provided in Table 2. All isolates displayed one or more enterotoxin genes, and seven strains carried all three enterotoxin genes (*nheA/B*, *hblD/A*, and *cytK*). One cereulide-producer was isolated out of a PIF product collected on retail level.

3.2 Affiliation of isolates to *panC* groups and visualization of genetic relatedness in SplitsTree

The 43 isolates represented five different *panC* groups (Table 3). No representatives of group I and VI could be found. All *panC* group VII isolates were positive for *cytKI*. The *B. thuringiensis* isolate belonged to *panC* group III. Most of the strains were affiliated with group III, including the strain positive for *ces* (strain P22). In addition to *panC* typing was performed. The similarity of *panC* nucleotide sequences of the isolates was depicted by a SplitsTree (Figure 1). The isolates formed clusters consistent with the results of *panC* typing. Apart from *B. cytotoxicus* isolates, all isolates from mashed potato powder formed a highly homogeneous group and belonged to the cluster exclusively comprising *panC* group III isolates, while strains that originated from PIF and fruit powder showed a higher degree of heterogeneity.

3.3 Cytotoxicity testing of *B. cytotoxicus* isolates

Cytotoxicity in a Vero cell assay was determined for all *B. cytotoxicus* isolates. Seven out of nine isolates exhibited no detectable cytotoxic effect. One isolate showed very low cytotoxicity and another isolate exhibited cytotoxicity 4.5 times as toxic as the highly toxic reference strain (Figure 2).

4. Discussion

The present study revealed a high prevalence of *B. cereus* group species in mashed potato powder and PIF products. *B. cereus* group species were detected in 92% of tested mashed potato powders. Based on varying sample sizes, prevalence rates for *B. cereus* in dehydrated potato products of 74% (Turner *et al.*, 2006) and 10 to 40% (King *et al.*, 2007) have been previously reported. The prevalence in PIF in the current study (78%) is similar to a large study from Becker *et al.* who stated that 70% of the powdered infant formula in

Germany were positive for *B. cereus s.l.* (Becker et al., 1994). Results obtained by *panC* typing were consistent with clusters formed by SplitsTree based on *panC* sequences. A correlation of toxin patterns and *panC* types could however not be seen, except for *panC* group IV, which exclusively comprised isolates positive for *nhe*, *hbl*, and *cytK2*. Toxin gene profiling of all isolates investigated in frame of this study revealed that all *B. cereus s.s.* harbor *nheA/B*, consistent with previous publications reporting that *nhe* is present in almost all *B. cereus s.s.* (Ehling-Schulz et al., 2011).

Only one *B. thuringiensis* strain was detected by screening for parasporal crystals (data not shown). However, this method may not be fully reliable, as tiny or irregular crystals can be missed (EFSA, 2016). The strain detected in our study was isolated from PIF and assigned to *panC* group III, consistent with previous assignments of *B. thuringiensis* to this *panC* group (Guinebretière et al., 2008). While there were no reports of *B. thuringiensis* in PIF, they are known to be a common contaminant of milk (Bartoszewicz et al., 2008). *B. thuringiensis*-based insecticides are used worldwide in agriculture and are highly effective against different groups of insects (Chattopadhyay et al., 2004) including the Colorado potato beetle - the most destructive insect pest of potato - that is also widespread in Switzerland (Wang et al., 2017). Still, no *B. thuringiensis* strains were detected in mashed potato powder samples investigated in this study.

The cluster analysis and *panC* typing revealed that most of the isolated strains belonged to group III, which has previously been suggested to harbor cytotoxic strains (Guinebretière et al., 2010). To date, outbreaks of emetic illness due to *B. cereus s.l.* have exclusively been associated with this *panC* type (Guinebretière et al., 2010). Notable, apart from *B. cytotoxicus* isolates, this cluster included all isolates obtained from mashed potato powders, while isolates originating from PIF showed much higher phylogenetic heterogenicity.

Mashed potatoes are often served in child-care institutions or hospitals, where they are likely to be held at temperatures promoting growth of germinated bacteria (Turner *et al.*, 2006), before being served to particularly vulnerable groups of humans. To prevent becoming ill with diarrheal or emetic syndrome when eating mashed potatoes, it is essential to keep the food above 60°C or to dispose of it within 2 h as Turner *et al.* have shown (Turner *et al.*, 2006).

FAO and WHO classified *B. cereus s.l.* as an organism of concern in PIF with regard to the strength of evidence of a causal association between the presence of the microorganism in PIF and illness in infants (FAO/WHO, 2006). Indeed, several studies reported high contamination levels of *B. cereus s.l.* in PIF (Rowan *et al.*, 1997; Zhang *et al.*, 2017). Due to the increasing numbers of *B. cereus* infections in infants (Gaur *et al.*, 2001; Hilliard *et al.*, 2003; Wang *et al.*, 2009), EFSA suggests the numbers of *B. cereus s.l.* spores in PIF should be as low as possible (EFSA, 2005). Lequin *et al.* reported three preterm infants with fatal hemorrhagic meningoencephalitis due to *B. cereus* infections (Lequin *et al.*, 2005).

Although *ces*-positive strains have been rarely reported from food samples, their occurrence often resulted in fatalities (Dierick *et al.*, 2005; Naranjo *et al.*, 2011; Takabe and Oya, 1976). In the present study, one cereulide-producer was isolated out of a PIF product which is consistent with other studies (Andersson *et al.*, 2004; Zhang *et al.*, 2017). The presence of a cereulide-producing strain in PIF raises concern, given the fact that this toxin can be preformed in the reconstituted PIF. It was shown by Shaheen *et al.* that PIF containing cereal as well as dairy ingredients are especially conducive for cereulide production (Shaheen *et al.*, 2006).

In contrast to mashed potato powders, no *B. cytotoxicus* could be detected in PIF. Nine isolates were found in mashed potato powder that harbored the *cytK1* variant, which is known to have necrotic and hemolytic activity and whose toxic potential is stated to be higher compared to *cytK2* (Fagerlund *et al.*, 2004). Up to now, only few strains of *B. cytotoxicus*

have been further characterized (Guinebretière *et al.*, 2013). This low number could be due to the fact that isolated *B. cereus s.l.* strains are normally summarized under the term of “presumptive *B. cereus*” comprising all different group members (Ehling-Schulz and Messelhäusser, 2013). The present study revealed a high prevalence of *B. cytotoxicus* in mashed potato powders. This is in accordance with the study of Contzen *et al.* who found a prevalence of 88% in mashed potato powder, flakes and granules (Contzen *et al.*, 2014). All nine *B. cytotoxicus* isolated in the present study could be assigned to *panC* group VII, which is known to exclusively comprise *B. cytotoxicus* (Guinebretière *et al.*, 2008, 2013). Depicting the isolates in a SplitsTree has shown that *B. cytotoxicus* isolates (M12-M20) represent a very remote cluster within the *B. cereus* group, consistent with other phylogenetic analyses using MLST (Fagerlund *et al.*, 2007; Sorokin *et al.*, 2006). It stays unclear why *B. cytotoxicus* has been mostly associated with mashed potato powders (Contzen *et al.*, 2014) or potato purée (Guinebretière *et al.*, 2013), considering that also PIF contain a high level of carbohydrates like starch, sucrose or lactose. Contzen *et al.* hypothesized that soil may be the source of contamination for mashed potato powders, as they had found *B. cytotoxicus* on a raw potato (Contzen *et al.*, 2014).

The results of the performed cytotoxicity assays in this study suggest that there are few strains which are highly cytotoxic, and which could lead to food poisoning outbreaks, while most *B. cytotoxicus* seem to be non-toxic. However, up to now, cytotoxicity assays have – with one exception - only been performed with strains related to food poisoning cases, thus leading to an overestimation of the cytotoxicity of *B. cytotoxicus* (Fagerlund *et al.*, 2007). The results of the present study support the assumption of Fagerlund *et al.* that harboring the *cytKI* gene is not a sufficient criterion for highly cytotoxic strains (Fagerlund *et al.*, 2007). Fagerlund *et al.* have also shown that the different levels of expression of *cytKI* could not be due to differences in the PlcR-PapR quorum sensing system, which acts as key transcriptional regulator for extracellular virulence factors in *B. cereus* group strains (Fagerlund *et al.*, 2007).

Furthermore, YvrGH and YvfTU two-component systems have also been studied and neither seem to be responsible for the differences in the expression of *cytK1*.

In conclusion, this study shows that *B. cereus s.l.* in mashed potato powders as well as PIF pose a potential food safety risk. Further research is needed to extend the hitherto very limited knowledge on the ecological niches of *B. cytotoxicus* and mechanism of its cytotoxicity. Due to the ubiquity, resistance, and persistence of *B. cereus s.l.* and colonization of processing facilities with spores (Carlin, 2011), contamination of food products is almost impossible to avoid. It is therefore essential that producers uphold highest quality control standards, while consumers should assure good practices such as proper holding times and storage temperatures to protect especially vulnerable consumer groups such as infants or hospital inpatients.

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Competing interests

The authors declare that they have no competing interests.

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TABLES AND FIGURES

Table 1: Primers used in this study.

Target gene	Primer	Primer sequence (5' → 3')	Reference
<i>panC</i>	panC_Cyto_for	CGTTATCCAAGGGATATAAAGCGA	This study
	panC_Cyto_rev	TCTACATAATCAACTATACCGTTTG	This study
<i>panC</i>	panC_fwd	CGATATCCTCGTGATATTGATAGA	Sorokin <i>et al.</i> (2006)
	panC_rev	TCCGCATAATCTACAGTGGCTTTC	Sorokin <i>et al.</i> (2006)
<i>nhe</i>	NA2F	AAGCIGCTCTTCGIATTC	Ehling-Schulz <i>et al.</i> (2006)
	NB1R	ITIGTTGAAATAAGCTGTGG	Ehling-Schulz <i>et al.</i> (2006)
<i>hbl</i>	HD2F	GTAAATTAIGATGAICAATTTC	Ehling-Schulz <i>et al.</i> (2006)
	HA4R	AGAATAGGCATTCATAGATT	Ehling-Schulz <i>et al.</i> (2006)
<i>ces</i>	CesF1	GGTGACACATTATCATATAAGGTG	Ehling-Schulz <i>et al.</i> (2006)
	CesR2	GTAAGCGAACCTGTCTGTAACAACA	Ehling-Schulz <i>et al.</i> (2006)
<i>cytK1</i>	CK1F	CAATTCCAGGGGCAAGTGTC	Guinebretiere <i>et al.</i> (2006)
	CK1R	CCTCGTGCATCTGTTTCATGAG	Guinebretiere <i>et al.</i> (2006)
<i>cytK2</i>	CK2F	CAATCCCTGGCGCTAGTGCA	Guinebretiere <i>et al.</i> (2006)
	CK2R	GTGIAGCCTGGACGAAGTTGG	Guinebretiere <i>et al.</i> (2006)

Table 2: Toxin genes detected by PCR in a total of 43 *B. cereus s.l.* isolates collected from powdered infant formula (PIF), mashed potato powder, and fruit powder.

	<i>nhe</i>	<i>hbl</i>	<i>cytK1</i>	<i>cytK2</i>	<i>ces</i>
PIF ^P isolates (n = 11)	11	4	0	8	0
PIF ^R isolates (n = 8)	8	1	0	5	1
Mashed potato powder isolates (n = 20)	12	2	9	6	0
Fruit powder isolates (n = 4)	4	2	0	4	0

PIF^P Samples obtained at the level of production

PIF^R Samples obtained at retail level

Table 3: Assignment of 43 *B. cereus s.l.* isolates originating from different food sources to *panC* groups

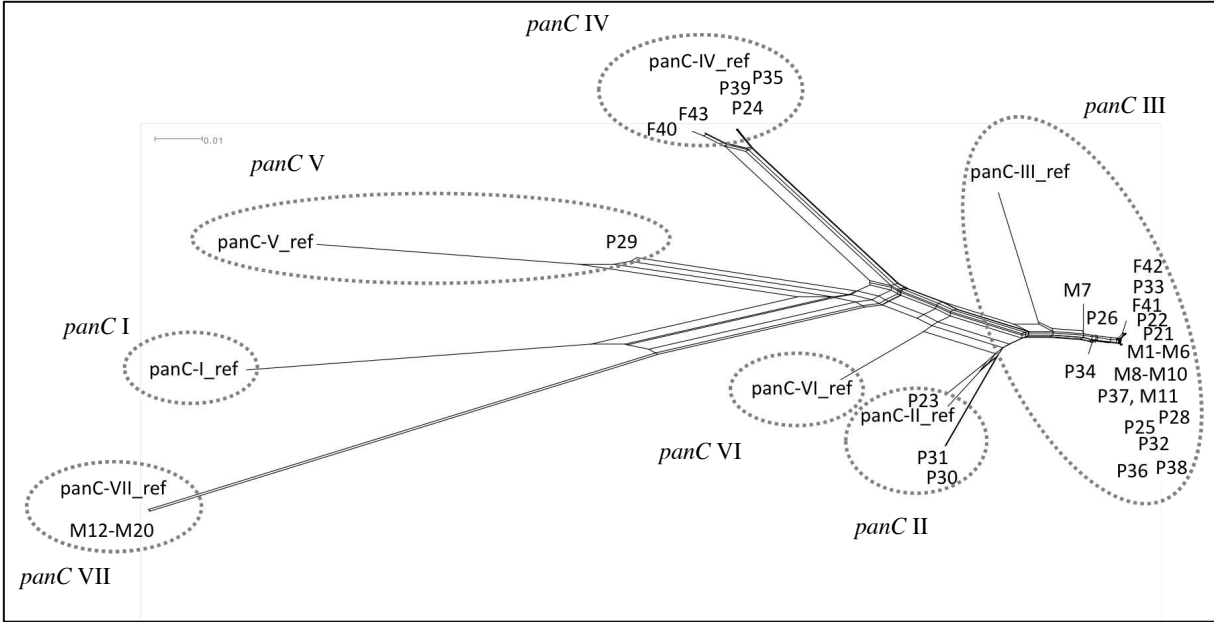
<i>panC</i> group	PIF ^P isolates (n = 11)	PIF ^R isolates (n = 8)	Mashed potato powder isolates (n = 20)	Fruit powder isolates (n = 4)
II	2	1	0	0
III	6	6	10	2
IV	2	1	0	2
V	1	0	0	0
VII	0	0	9	0
NS	0	0	1	0

NS = no assignment to any of the *panC* groups I-VII.

PIF^P Samples obtained at the level of production

PIF^R Samples obtained at retail level

Figure 1: SplitsTree depicting the degree of similarity of the *panC* sequences. (a) Overview over the full SplitsTree depicting all isolates as well as one reference strain per *panC* type (*panC* type I: DSM 12442; *panC* type II: WSBC10311; *panC* type III: Ames; *panC* type IV: ATCC 14579; *panC* type V: BCT-7112; *panC* type VI: WSBC 10204; *panC* type VII: NVH391-98); (b) Detail zooming in on the region depicting the *panC* type III cluster, while omitting isolates assigned to other *panC* groups. M = isolate originating from mashed potato powder, P = isolate originating from PIF, F = isolate originating from fruit powder.



(b)

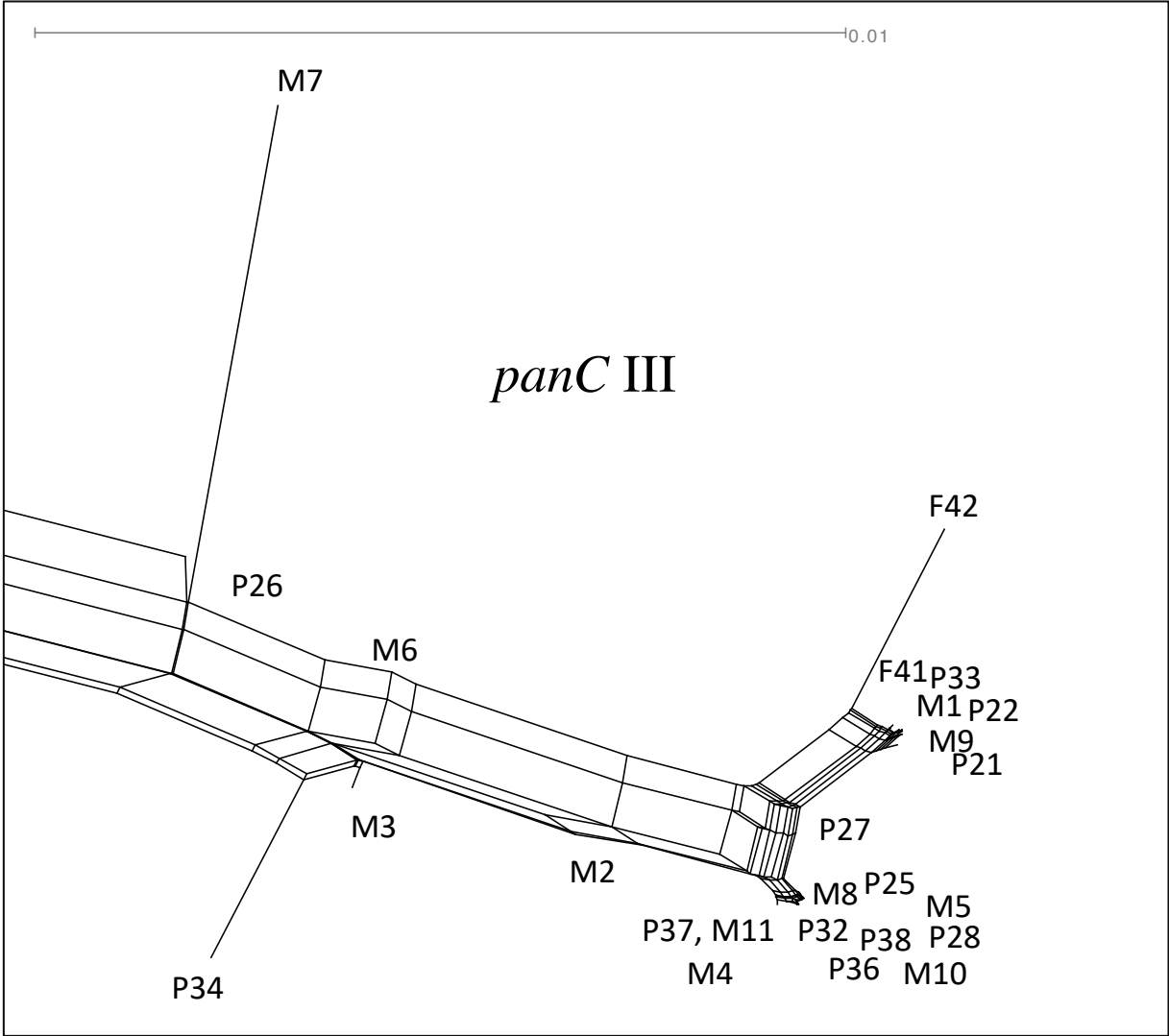


Figure 2: Reciprocal cytotoxicity titers of *B. cytotoxicus* isolates M12-M20 and a reference strain for high level toxin production (food poisoning strain *B. cereus* NVH 0075-95). Values indicated are based on supernatants tested in two Vero cell cytotoxicity assays with each dilution of the supernatant tested in duplicate. Error bars represent one standard deviation of the mean.

